Treatment of lactating dairy cows with gonadotropin-releasing hormone before first insemination during summer heat stress

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ABSTRACT

The objectives of the experiments were to compare ovarian responses, pregnancy per artificial insemination, and pattern of insemination of 2 estrus detection-based presynchronization protocols before first artificial insemination (AI) during heat stress. In experiment 1, primiparous lactating dairy cows ($n = 1,358$) from 3 dairies were assigned randomly to 2 treatments at 60 ± 3 ($\pm$ SD) DIM (study d 0): (1) treatment with 100 μ g of GnRH on study d 0 (Gpresynch), or (2) no treatment on study d 0 (control). In experiment 2, multiparous lactating dairy cows ($n = 1,971$) from 3 dairies were assigned randomly to 2 treatments at 49 ± 3 ($\pm$ SD) DIM (study d 0), similar to experiment 1. In both experiments, PGF_{2 α} injections were administered 14 d apart starting on study d 7 for all cows. Cows not inseminated after detection of estrus were submitted to a timed artificial insemination protocol at study d 35. In a subgroup of cows from 2 dairies, concentrations of progesterone were determined from blood samples collected on study d 0 and 7. Furthermore, ovaries were examined by ultrasonography on study d -14 , 0, and 7 to determine cyclic status and ovulation in response to GnRH treatment. In experiment 1, progesterone concentration was not different on d 0, but progesterone was increased for Gpresynch compared with control cows on study d 7 (3.6 ± 0.3 vs. 2.7 ± 0.4 ng/mL), respectively. Ovulation risk from study d 0 to 7 was increased for Gpresynch compared with control (50.6 vs. 15.2%). Control cows were inseminated at a faster rate than Gpresynch cows [adjusted hazard ratio (AHR) = 0.89, 95% confidence interval = 0.80 to 1.00], and the interaction between treatment and dairy affected pregnancy per artificial insemination at 36 and 94 d post-artificial insemination. In experiment 2, concentrations of progesterone did not differ on study d 0 or 7,

despite ovulation risk from study d 0 to 7 being greater in Gpresynch than control cows (46.9 vs. 23.8%). The interaction between treatment and dairy affected hazard of insemination with Gpresynch cows from dairy 1 (AHR = 1.21; 1.05 to 1.41) being inseminated faster than control cows. Hazard of pregnancy was affected by treatment because Gpresynch cows became pregnant at a faster rate than control cows (AHR = 1.25; 1.04 to 1.50). In conclusion, GnRH-based presynchronization protocols initiated before the end of the voluntary waiting period may have benefits in reproductive efficiency of estrus detection-based programs during heat stress. In addition, treatment with GnRH decreased the prevalence of anovular cows at the initiation of PGF_{2 α} injections.

Key words: dairy cow, estrus detection, gonadotropin-releasing hormone, presynchronization

INTRODUCTION

Reproductive efficiency in dairy cattle has steadily declined during the past 45 yr (Lucy, 2001), with declines in reproductive performance greatest during summer because of heat stress (De Rensis and Scaramuzzi, 2003). Poor oocyte and embryo quality, decreased fertilization rates (Sartori et al., 2002), and increased embryonic and fetal mortality (Santos et al., 2004) partly explain the decreased fertility in cows exposed to heat stress during summer. The direct effect of heat stress on the gonads is one mechanism by which heat stress affects fertility. Heat-stressed dairy cows have altered hormone production and metabolism, which induce changes in the follicular micro-environment (Leroy et al., 2011).

The progesterone environment in which a follicle develops is believed to affect the quality of oocyte, and consequently, embryo quality (Wiltbank et al., 2014). Rivera et al. (2011) demonstrated that super-stimulated cows that had greater progesterone concentration before AI had a greater percentage of excellent/good and fair embryos recovered 6.5 d after AI, compared with cows with a lesser concentration of progesterone. Furthermore, increased pregnancy per AI (**P/AI**) was

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detected in cows with follicles growing under greater concentration of progesterone before AI (Denicol et al., 2012). Although greater concentration of progesterone before AI has been studied in cows submitted to timed AI (TAI) protocols, little is known about greater concentration of progesterone in cows inseminated based on estrus (no TAI). Furthermore, others (Rivera et al., 2011; Denicol et al., 2012) did not investigate whether greater concentration of progesterone during dominant follicle selection had an effect during periods of summer heat stress, a time in which improving oocyte and embryo quality would be desired.

Several studies demonstrated that GnRH-PGF_{2α}-based presynchronization protocols for first TAI increased P/AI compared with only using PGF_{2α} in lactating dairy cows (Stevenson et al., 2012; Ayres et al., 2013; Dirandeh et al., 2015). Cows exposed to presynchronization with PGF_{2α} followed in 3 d by GnRH (PG-3-G), 7 d before Ovsynch, had greater P/AI compared with PGF_{2α}-based presynchronization (Stevenson et al., 2012). The percentage of cows bearing a corpus luteum (CL) and progesterone concentration ≥ 1 ng/mL was greater in the PG-3-G cows than the PGF_{2α}-based presynchronized controls at the time of the first GnRH injection of the TAI protocol (Stevenson and Pulley, 2012), indicating improved synchrony of the estrous cycle. Improved fertility of cows synchronized with a GnRH-PGF_{2α}-based presynchronization protocol compared with a PGF_{2α}-based presynchronization protocol was partly related to the increased concentration of progesterone during dominant follicle selection (Ayres et al., 2013).

A study conducted during summer demonstrated that synchronizing the first TAI with Double-Ovsynch, a GnRH-PGF_{2α}-based presynchronization protocol, improved fertility in dairy cows compared with the Presynch-Ovsynch, PGF_{2α}-based presynchronization protocol (Dirandeh et al., 2015). Although concentrations of progesterone were not evaluated, a greater proportion of Double-Ovsynch cows had a CL present at the first GnRH of the TAI protocol, which may have resulted in greater concentration of progesterone during dominant follicle selection before AI (Dirandeh et al., 2015). The reported improved fertility of cows submitted to PG-3-G (Stevenson and Pulley, 2012) and Double-Ovsynch (Dirandeh et al., 2015) during summer may be a result of increased concentration of progesterone as previously suggested (Ayres et al., 2013). In contrast, the improved fertility may be consequence of increased fertility of anovular cows. The additional and multiple injections of GnRH in the PG-3-G and Double-Ovsynch protocols, respectively, are likely to induce ovulation during the protocol, which is believed to be beneficial

for anovular cows. Because anovulation before first AI is associated with poor fertility (Bisinotto et al., 2010), inducing ovulation with an injection of GnRH during presynchronization protocols may have an additional benefit to anovular cows compared with protocols based only on PGF_{2α}. Despite the potential benefits of using GnRH in presynchronization protocols, herds that strive to inseminate cows based on estrus detection may choose to use PGF_{2α}-based presynchronization protocols in lieu of GnRH-PGF_{2α}-based protocols because GnRH suppresses signs of estrus (Mendonça et al., 2012; Bruno et al., 2013; Chebel et al., 2013). Thus, a presynchronization protocol that induces estrus expression and ovulation of anovular cows, and increases concentration of progesterone during dominant follicle selection, may improve reproductive efficiency during summer heat stress in herds that submit cows to AI largely based on estrus detection.

We hypothesized that cows treated with GnRH before an estrus detection-based presynchronization protocol would induce ovarian cyclicity in anovular cows, increase concentration of progesterone, and improve P/AI of heat-stressed cows. The objectives of the experiments were to compare ovarian response, P/AI, and pattern of insemination of 2 estrus detection-based presynchronization protocols for first insemination during summer heat stress.

MATERIALS AND METHODS

Cows, Housing, and Feeding

These experiments were conducted in 3 commercial dairy herds. Dairy 1 was located in the panhandle of Oklahoma, and dairies 2 and 3 were located in southwest Kansas. Lactating Holstein dairy cows in the 3 herds were enrolled in the experiments from June 2014 through September 2014. Cows from dairy 1 were housed in dry-lot corrals without shade and were milked thrice daily with a projected 305-d milk yield of 8,400 kg. Cows from dairy 2 were housed in dry-lot corrals with shade and were milked twice daily with a projected 305-d milk yield of 8,904 kg. Cows from dairy 3 were housed in free-stall barns equipped with fans and sprinklers, with access to a dirt exercise lot, and milked thrice daily with a projected 305-d milk yield of 9,017 kg. The 3 herds were fed a TMR once daily with ad libitum access to feed and water. Milk yields were recorded at each milking using a parlor management software program (DairyPlan C21, GEA Farm Technologies, Naperville, IL). Average daily milk yield during the week before enrollment was extracted from the on-farm software.

Temperature and Relative Humidity Measurements

Temperature and relative humidity measurements were collected from the closest official meteorological stations to the dairy farms. Temperature and relative humidity measurements for dairy 1 was collected from a meteorological station located in Clayton, New Mexico, and measurements for dairy 2 and 3 were collected from a meteorological station located in Liberal, Kansas. Using the measurements collected, hourly temperature-humidity index (**THI**) was calculated using the following formula (NOAA, 1976): $T - [0.55 - (0.55 \text{ RH}/100) \times (T - 58)]$; where T and RH are dry bulb temperature (°F) and relative humidity, respectively.

Experiment 1—Primiparous Cows

For experiment 1, weekly cohorts of primiparous cows were enrolled in the study at 60 ± 3 DIM (study d 0). Cows (n = 1,358) were assigned randomly based on ear tag identification number to 2 treatments: (1) treatment with 100 µg of GnRH (Factrel, Zoetis Inc., Florham Park, NJ; **Gpresynch**), or (2) no treatment (control) at study d 0. At study d 7, all cows were presynchronized with two 25-mg injections of PGF_{2α} (dinoprost tromethamine; 5 mL of Lutalyse, Zoetis Inc.) injections given 14 d apart. Estrus detection was performed once daily based on tail paint removal. Cows detected in estrus were eligible to be inseminated after 53 DIM. Once inseminated, cows received no further injections of the synchronization protocol. Cows not

inseminated by study d 35 were enrolled in the Co-synch-72 protocol for TAI (Figure 1).

Experiment 2—Multiparous Cows

Weekly cohorts of multiparous cows were enrolled in the study at 49 ± 3 DIM (study d 0). Cows (n = 1,971) were assigned randomly based on ear tag identification number to 2 treatments: (1) treatment with 100 µg of GnRH (Gpresynch); or (2) no treatment (control) at study d 0. At study d 7, all cows received two 25-mg PGF_{2α} injections given 14 d apart. Estrus detection was performed once daily based on tail paint removal. Cows detected in estrus were eligible to be inseminated after 53 DIM. Once inseminated, cows received no further injections of the synchronization protocol. Cows not inseminated by study d 35 were enrolled in the Co-synch-72 protocol for TAI (Figure 1).

Blood Sampling, Progesterone, and Ovarian Ultrasonography

In a subgroup of cows (experiment 1 = 225 and experiment 2 = 394) from dairies 2 and 3, ovaries were examined by ultrasonography on study d -14, 0, and 7. Number of follicles ≥10 mm in diameter and presence of a CL ≥20 mm in diameter in each ovary were recorded. Cyclic status was determined by the presence of a CL at study d -14 or 0. Cows were considered to have ovulated to the GnRH injection if they had a

Figure 1. Schematic representation of experimental procedures for experiments 1 and 2. Primiparous cows in experiment 1 were enrolled in the study at 60 ± 3 ($\pm$ SD) DIM (study d 0). Multiparous cows in experiment 2 were enrolled at 49 ± 3 DIM (study d 0). In both experiments, cows became eligible to be inseminated at 53 DIM. On study d 0, cows were assigned randomly to receive either 100 µg of GnRH (Gpresynch) or no GnRH (control). Cows were submitted to a presynchronization with PGF_{2α} 14 d apart at study d 7. Cows inseminated received no further treatment, whereas cows not inseminated by study d 35 were enrolled in the Cosynch-72 protocol [GnRH -7 d-PGF_{2α} -3 d-GnRH+timed artificial insemination (TAI)] for TAI. Ovarian ultrasonography examinations (US) were performed on study d -14, 0, and 7, and blood collection (BC) was performed on study d 0 and 7.

follicle ≥ 10 mm on d 0 and a new CL present in the same ovary on d 7.

In a subgroup of cows (experiment 1 = 224 and experiment 2 = 394) from dairies 2 and 3, blood samples were collected on d 0 and 7 to determine concentration of progesterone. Blood samples were collected from a coccygeal vessel into evacuated tubes containing K2 EDTA (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ). Following collection, blood samples were placed on ice until centrifugation at $1,200 \times g$ for 15 min for plasma separation. Plasma samples were frozen and stored at -20°C until analysis. Concentration of progesterone was determined using a solid-phase RIA kit (Coat-a-Count, Siemens Medical Solutions Diagnostics, Los Angeles, CA) previously validated (Stevenson et al., 2012). Samples were assayed in duplicate with intra-assay and inter-assay coefficients of 8.0 and 8.8%, respectively. Cows were classified as having concentration of progesterone ≥ 1 ng/mL or as having concentration of progesterone < 1 ng/mL at study d 0 and 7 for statistical analysis.

Pregnancy Diagnosis

Cows in both experiments were submitted for pregnancy diagnosis at 36 ± 3 d after AI by transrectal ultrasonography based on detection of fluid, and presence of an embryo with a heartbeat. At dairies 1 and 2, pregnancy was reconfirmed 94 ± 3 d after AI. At dairy 3, cows diagnosed pregnant were not re-examined to determine pregnancy loss.

Statistical Analyses

The experiment was a completely randomized design with randomization based on ear tag identification number. Cows with even ear tag identification number were assigned to the Gpresynch treatment and cows with odd ear tag identification number were assigned to the control. In experiment 1, a sample size of 650 experimental units in each treatment was expected to detect a statistical difference with a 6.5% unit difference in P/AI, when P/AI ranges from 30 to 37% ($\alpha = 0.05$; $\beta = 0.20$; one-tailed test). The same number of experimental units was expected to detect a difference in 1.7 d difference in days to AI (SD = 12.0; $\alpha = 0.05$; $\beta = 0.20$; one-tailed test). In experiment 2, a sample size of 950 experimental units per treatment was needed to detect a statistical difference with a 5% unit difference in P/AI when P/AI ranges from 25 to 30% ($\alpha = 0.05$; $\beta = 0.20$; one-tailed test). In addition, it was expected to detect a statistical difference in 1.3 d difference in days to AI (SD = 11.7; $\alpha = 0.05$; $\beta = 0.20$; one-tailed test).

Dichotomous outcomes were analyzed using the LOGISTIC procedure of SAS version 9.3 (SAS Institute Inc., Cary, NC). Continuous outcomes were analyzed by ANOVA using the GLM procedure of SAS. Models for analysis of P/AI and pregnancy loss included the following fixed effects in the models: treatment, dairy, and the interactions between treatment and dairy. The study timeline was categorized into 4 time periods to investigate potential treatment differences in P/AI and percentage of cows inseminated at specific time points: period 1 = study d 0 to 7; period 2 = study d 8 to 21; period 3 = study d 22 to 35; and period 4 = study d 36 to 45.

Variables included in the models for analysis of progesterone concentration, percentage of cows with progesterone ≥ 1 ng/mL, and ovulation risk were treatment, dairy, cyclic status, and interactions between treatment and dairy and treatment and cyclic status. Backward stepwise elimination was performed to remove variables ($P > 0.10$). Treatment and dairy were forced to remain in the models.

The rate at which cows were inseminated or became pregnant were analyzed by the Cox proportional hazard ratio using the PHREG procedure of SAS. Variables were removed using the stepwise backward elimination function based on the Wald statistic criterion when $P > 0.10$. Survival analysis for time to insemination and pregnancy were performed using the LIFETEST procedure of SAS. Statistical significance was determined as $P \leq 0.05$ and statistical tendencies as $0.05 < P \leq 0.10$.

RESULTS

In experiment 1, 57 primiparous cows were culled or deemed not eligible to be inseminated before AI, and 24 primiparous cows were culled before pregnancy diagnosis. In experiment 2, 103 and 40 multiparous cows were culled or deemed not eligible to be inseminated before AI, and culled before pregnancy diagnosis, respectively.

Milk Production and Temperature-Humidity Index

In experiment 1, projected 305-d milk ($7,597 \pm 28$ kg; $P = 0.30$) and average milk yield 1 wk before enrollment (30.0 ± 0.2 kg/d; $P = 0.51$) did not differ between treatments. Furthermore, in experiment 2, projected 305-d milk ($9,638 \pm 29$ kg; $P = 0.34$) and average milk yield 1 wk before enrollment (43.4 ± 0.2 kg/d; $P = 0.42$) did not differ between treatments.

Average daily maximum THI from June 2014 to September 2014 for dairy 1 was 88.9, and was 94.7 for dairies 2 and 3. Furthermore, the average daily minimum THI for dairy 1 was 60.9 and the average daily

minimum THI was 66.4 for dairies 2 and 3. Average daily maximum and minimum temperature were 29.2 and 15.6°C for dairy 1, and 31.5 and 17.5°C for dairies 2 and 3, respectively. Average daily maximum and minimum relative humidity were 80.5 and 30.4% for dairy 1, and 79.2 and 35.7% for dairies 2 and 3, respectively. Mean daily percentage of hours with THI ≥ 72 was 50.4% for dairy 1, and 70.9% for dairies 2 and 3.

Experiment 1—Primiparous Cows

Concentration of Progesterone and Ovarian Response. Concentration of progesterone and percentage of cows with progesterone concentration ≥ 1 ng/mL on d 0 did not ($P \geq 0.57$) differ between treatments (Table 1). Furthermore, the percentage of noncyclic cows ($P = 0.17$) and cows with a CL present on d 0 ($P = 0.12$) did not differ between control and Gpresynch cows (Table 1).

Concentration of progesterone on d 7 was greater ($P < 0.01$) in Gpresynch than control cows (3.6 ± 0.3 vs. 2.7 ± 0.4 ng/mL). In addition, the percentage of cows with progesterone concentration ≥ 1 ng/mL on d 7 was

greater ($P < 0.01$) in Gpresynch than control cows (79.1 vs. 58.0%). The Gpresynch cows had a greater ($P < 0.01$) percentage of cows with a CL present on d 7 (91.7 vs. 70.9%). Risk of ovulation from d 0 to 7 was increased ($P < 0.01$) for Gpresynch cows compared with control cows (50.6 vs. 15.2%). Furthermore, treatment with GnRH decreased ($P = 0.02$) the percentage of cows that did not have a CL present at any of the ultrasound examinations conducted on study d -14, 0, and 7 (2.7 vs. 10.3%). No interactions ($P = 0.74$) were detected between dairy and concentration of progesterone, dairy and percentage of cows with progesterone concentration ≥ 1 ng/mL, and dairy and percentage of cows with a CL present on d 7 (Table 1).

Pregnancy per AI and Pregnancy Loss. Treatment did not ($P = 0.48$) affect overall P/AI 36 d after AI; however, an interaction was detected ($P = 0.01$) between treatment and dairy for P/AI (Table 2). Furthermore, the interaction between treatment and dairy affected ($P = 0.01$) P/AI at d 94 after AI. In dairy 1, P/AI was greater ($P = 0.03$) for Gpresynch cows than control cows at 36 d after AI. In contrast, in dairy 2, control cows had greater ($P = 0.05$) P/AI at d 36

Table 1. Concentration of progesterone, presence of corpus luteum (CL), and ovulation risk for primiparous and multiparous cows enrolled in experiment 1 and 2, respectively¹

Item	Treatment (T)		P-value		
	Control	Gpresynch	T	Dairy	T $\times$ dairy
Experiment 1: primiparous cows	(n = 123)	(n = 102)			
Study d 0					
Noncyclic status, ² %	29.5	32.5	0.17	0.97	0.68
Cows bearing a CL, %	71.5	61.8	0.12	0.83	0.67
Progesterone concentration, ng/mL	2.3 ± 0.3	2.5 ± 0.3	0.57	0.83	0.16
Progesterone concentration ≥ 1 ng/mL, %	59.5	53.9	0.74	0.30	0.40
Study d 7					
Cows bearing a CL, %	70.9	91.7	<0.01	0.92	0.77
Progesterone concentration, ng/mL	2.7 ± 0.4	3.6 ± 0.3	<0.01	0.07	0.75
Progesterone concentration ≥ 1 ng/mL, %	58.0	79.1	<0.01	0.20	0.74
Ovulation risk, ³ %	15.2	50.6	<0.01	0.32	0.34
No CL present, ⁴ %	10.3	2.7	0.02	0.26	0.99
Experiment 2: multiparous cows	(n = 198)	(n = 196)			
Study d 0					
Noncyclic status, ² %	12.8	19.0	0.15	<0.01	0.87
Cows bearing a CL, %	74.8	73.0	0.99	0.41	0.40
Progesterone concentration, ng/mL	3.4 ± 0.3	3.8 ± 0.3	0.30	0.13	0.63
Progesterone concentration ≥ 1 ng/mL, %	63.2	66.8	0.43	<0.01	0.07
Study d 7					
Cows bearing a CL	83.3	95.6	<0.01	0.29	0.58
Progesterone concentration, ng/mL	4.4 ± 0.3	4.8 ± 0.3	0.34	0.42	0.63
Progesterone concentration ≥ 1 ng/mL, %	76.4	85.8	0.03	0.93	0.48
Ovulation risk, ³ %	23.8	46.9	<0.01	0.85	0.50
No CL present, ⁴ %	5.8	1.5	0.04	0.14	0.88

¹Cows were either treated (Gpresynch) or not treated (control) with GnRH before presynchronization with 2 injections of PGF_{2α} given 14 d apart.

²Percentage of cows not bearing a CL on study d -14 to 0.

³Percentage of cows that had an ovulatory response from study d 0 to 7.

⁴Percentage of cows that did not have a CL present on study d -14, 0, and 7.

Table 2. Pregnancy per AI (P/AI) and pregnancy loss for treated (Gpresynch) and not treated (control) primiparous cows for experiment 1^{1,2}

Item	Treatment (T)		P-value		
	Control	Gpresynch	T	Dairy	T × dairy
Overall	(n = 655)	(n = 622)			
P/AI on d 36 ± 3, %	32.1	31.8	0.48	0.69	0.01
P/AI on d 94 ± 3, %	27.8	29.8	0.97	0.62	0.01
Pregnancy loss, %	8.2	6.9	0.72	0.51	0.71
Dairy 1	(n = 283)	(n = 255)			
P/AI on d 36 ± 3, %	27.6	36.1	0.03	—	—
P/AI on d 94 ± 3, %	25.5	33.5	0.04	—	—
Pregnancy loss, %	6.6	6.6	0.99	—	—
Dairy 2	(n = 163)	(n = 156)			
P/AI on d 36 ± 3, %	35.6	25.6	0.05	—	—
P/AI on d 94 ± 3, %	31.9	23.9	0.11	—	—
Pregnancy loss, %	10.3	7.5	0.63	—	—
Dairy 3	(n = 209)	(n = 211)			
P/AI on d 36 ± 3, %	35.4	31.3	0.37	—	—
P/AI on d 94 ± 3, %	—	—	—	—	—
Pregnancy loss, %	—	—	—	—	—

¹Primiparous cows were either treated with GnRH (Gpresynch) or not treated (control) before presynchronization with 2 injections of PGF_{2α} given 14 d apart.

²Data of P/AI at d 94 after AI and pregnancy loss only pertain to dairies 1 and 2.

post-AI compared with Gpresynch cows. In dairy 3, no difference ($P = 0.37$) between treatments was detected in P/AI at 36 d after AI. Similar results were observed for cows inseminated during period 2, in which an interaction between treatment and dairy affected ($P = 0.04$) P/AI 36 d after AI. In contrast, no differences

($P > 0.50$) were detected in other periods (Table 3). Pregnancy loss from d 36 to 94 post-AI did not differ ($P = 0.72$) between treatments (7.6%; Table 2).

Pattern of Insemination and Hazard of Pregnancy. During period 1, a greater ($P < 0.01$) percentage of control cows were inseminated than Gpresynch

Table 3. Pregnancy per artificial insemination (AI) and percentage of primiparous and multiparous cows inseminated by period for experiment 1 and experiment 2, respectively¹

Item	Treatment (T)		P-value		
	Control	Gpresynch	T	Dairy	T × dairy
Experiment 1: primiparous cows					
Percentage inseminated	(n = 670)	(n = 631)			
Period 1	25.5 (171/670)	9.4 (59/631)	<0.01	0.34	0.43
Period 2	43.1 (289/670)	58.3 (368/631)	<0.01	<0.01	0.78
Period 3	17.2 (115/670)	19.0 (120/631)	0.45	0.30	0.40
Period 4	14.2 (95/670)	13.3 (84/631)	0.73	<0.01	0.19
Pregnancy per AI (%)	(n = 655)	(n = 622)			
Period 1	38.8 (66/170)	42.4 (25/59)	0.50	0.64	0.42
Period 2	32.7 (91/278)	33.2 (121/364)	0.73	0.88	0.04
Period 3	27.4 (31/113)	30.3 (36/119)	0.71	0.85	0.65
Period 4	23.4 (22/94)	20.0 (16/80)	0.73	0.85	0.12
Experiment 2: multiparous cows					
Percentage inseminated	(n = 936)	(n = 932)			
Period 1	12.6 (118/936)	7.9 (74/932)	<0.01	0.09	0.53
Period 2	54.9 (514/936)	62.5 (582/932)	<0.01	<0.01	<0.01
Period 3	21.4 (200/936)	19.2 (179/932)	0.24	<0.01	0.03
Period 4	11.1 (104/936)	10.4 (97/932)	0.45	<0.01	0.12
Pregnancy per AI (%)	(n = 911)	(n = 917)			
Period 1	21.9 (25/114)	28.8 (21/73)	0.32	0.86	0.67
Period 2	25.5 (128/502)	26.9 (154/572)	0.59	0.44	0.93
Period 3	36.8 (71/193)	26.0 (46/177)	0.03	0.97	0.76
Period 4	24.5 (25/102)	12.6 (12/95)	0.04	0.09	0.35

¹Cows were either treated (Gpresynch) or not treated (control) with GnRH before presynchronization with 2 injections of PGF_{2α} given 14 d apart.

cows (25.5 vs. 9.4%). In contrast, during period 2, a greater ($P < 0.01$) percentage of Gpresynch cows were inseminated compared with control cows (58.3 vs. 43.1%). During periods 3 and 4, no differences ($P > 0.45$) in percentage of cows inseminated were detected between control and Gpresynch cows (Table 3).

Hazard of insemination was affected ($P = 0.05$) by treatment because control cows were inseminated at a faster rate [adjusted hazard ratio (AHR) = 0.89, 95% CI = 0.80 to 1.00] than Gpresynch cows. Despite the difference observed in hazard of insemination, hazard to pregnancy did not ($P = 0.35$) differ between treatments (AHR = 0.91, CI = 0.74 to 1.11). The interaction between treatment and dairy did not ($P \geq 0.29$) affect the hazard of insemination or hazard to pregnancy. According to the survival analysis, the interval from study d 0 to insemination was greater ($P \leq 0.01$) for Gpresynch cows compared with control cows in dairies 2 and 3. In contrast, in dairy 1 no difference ($P = 0.30$) was detected between treatments. Mean days to AI ($\pm$ SEM) were 16.7 ± 0.8 d, 16.0 ± 1.0 , and 18.0 ± 1.0 d for control cows from dairies 1, 2, and 3, respectively. Mean days to AI were 16.6 ± 0.7 d, 19.0 ± 1.1 d, and 20.0 ± 0.9 d for Gpresynch cows from dairies 1, 2, and 3, respectively. Median days to AI were 11 d for both control and Gpresynch cows from all dairies.

Experiment 2—Multiparous Cows

Concentration of Progesterone and Ovarian Response. Concentration of progesterone, percentage

of cows with concentration of progesterone ≥ 1 ng/mL, and percentage of cows bearing a CL did not ($P \geq 0.30$) differ between treatments on study d 0 (Table 1). Furthermore, percentage of cows classified in the noncyclic status at study d 0 did not ($P = 0.15$) differ between treatments.

Risk of ovulation from d 0 to d 7 was greater ($P < 0.01$) for Gpresynch cows than control cows (46.9 vs. 23.8%). Although percentage of cows with concentration of progesterone ≥ 1 ng/mL was greater ($P = 0.03$) in Gpresynch than control cows at study d 7, concentration of progesterone on d 7 did not ($P = 0.34$) differ between treatments (Table 1). Among cows not inseminated from study d 0 to 7, a greater ($P = 0.04$) percentage of control cows did not have a CL present at any of the 3 ultrasound examinations performed on study d -14, 0, and 7 compared with Gpresynch cows (5.8 vs. 1.5%).

Pregnancy per AI and Pregnancy Loss. Overall P/AI at d 36 ($P = 0.33$) and d 94 ($P = 0.47$) after AI did not differ between treatments (Table 4). In contrast, pregnancy loss from d 36 to 94 after AI was greater ($P = 0.04$) in Gpresynch than control cows (11.2 vs. 4.6%). Furthermore, control cows had greater ($P < 0.04$) P/AI during period 3 (36.8 vs. 26.0%) and period 4 (24.5 vs. 12.6%) compared with Gpresynch cows (Table 2). In contrast, no differences were detected between treatments during periods 1 ($P = 0.32$) and 2 ($P = 0.59$; Table 2).

Pattern of Insemination and Hazard of Pregnancy. During period 1, a greater ($P < 0.01$) percent-

Table 4. The effect of treatment and dairy on pregnancy per AI (P/AI) at 36 ± 3 and 94 ± 3 d after AI and pregnancy loss of multiparous cows from experiment 2^{1,2}

Item	Treatment (T)		P-value		
	Control	Gpresynch	T	Dairy	T $\times$ dairy
Overall	(n = 911)	(n = 917)			
P/AI on d 36 ± 3 , %	27.3	25.4	0.33	0.49	0.84
P/AI on d 94 ± 3 , %	22.9	21.1	0.47	0.38	0.13
Pregnancy loss, %	4.6	11.2	0.04	0.42	0.11
Dairy 1	(n = 341)	(n = 335)			
P/AI on d 36 ± 3 , %	28.2	27.8	0.91	—	—
P/AI on d 94 ± 3 , %	20.0	21.8	0.59	—	—
Pregnancy loss, %	7.5	9.2	0.71	—	—
Dairy 2	(n = 327)	(n = 333)			
P/AI on d 36 ± 3 , %	26.9	24.0	0.39	—	—
P/AI on d 94 ± 3 , %	25.7	20.4	0.11	—	—
Pregnancy loss, %	2.4	13.2	0.01	—	—
Dairy 3	(n = 243)	(n = 249)			
P/AI on d 36 ± 3 , %	26.8	24.1	0.50	—	—
P/AI on d 94 ± 3 , %	—	—	—	—	—
Pregnancy loss, %	—	—	—	—	—

¹Multiparous cows were either treated (Gpresynch) or not treated (control) with GnRH before presynchronization with 2 injections of PGF_{2α} given 14 d apart.

²Data of P/AI at 94 d after AI and pregnancy loss only pertain to dairy 1 and 2.

Figure 2. Survival analysis for days to pregnancy for multiparous cows treated with GnRH (Gpresynch) or control before presynchronization in experiment 2. Median days to pregnancy were 12 and 11 d for control and Gpresynch cows, respectively. Mean days to pregnancy ($\pm$ SEM) were 18.3 ± 0.7 and 15.9 ± 0.7 d for control and Gpresynch cows, respectively ($P = 0.02$).

age of control cows were inseminated than Gpresynch cows (Table 3). In contrast, during period 2, a greater ($P < 0.01$) percentage of Gpresynch cows were inseminated compared with control cows. Furthermore, the interaction between treatment and dairy affected ($P < 0.01$) the percentage of cows inseminated in period 2. Such an interaction was observed because in dairy 1, a greater ($P < 0.01$) percentage of Gpresynch cows were inseminated compared with control cows (71.6 vs. 53.4%), whereas in dairies 2 and dairy 3, no differences ($P > 0.35$) were detected between treatments during this time period. During period 3, an interaction of treatment and dairy affected ($P = 0.03$) the percentage of cows inseminated. This interaction was observed because a greater ($P < 0.01$) percentage of control cows from dairy 1 were inseminated than Gpresynch cows (21.7 vs. 13.5%). In contrast, no differences ($P > 0.44$) between treatments were detected in the percentage of cows inseminated at dairies 2 and 3.

Treatment did not ($P = 0.41$) affect hazard of insemination, but an interaction between treatment and dairy was detected ($P = 0.03$). In dairy 1, Gpresynch cows (AHR = 1.21; CI = 1.05 to 1.41) were inseminated at a faster ($P = 0.01$) rate than control cows, but no differences ($P > 0.23$) were detected in dairy 2 (AHR = 0.91; CI = 0.78 to 1.06), and dairy 3 (AHR = 1.02; CI = 0.85 to 1.21). The survival analyses revealed similar results, in which no differences ($P \geq 0.15$) were detected between treatments in the interval from study

d 0 to insemination in dairies 2 and 3; however, in dairy 1, the interval from study d 0 to insemination was less ($P = 0.02$) for Gpresynch cows compared with control cows. Mean days ($\pm$ SEM) from study d 0 to AI for control cows were 17.2 ± 0.6 , 16.9 ± 0.6 , and 20.6 ± 0.8 d for dairies 1, 2, and 3, respectively. Mean days from study d 0 to AI for Gpresynch cows were 15.2 ± 0.5 , 17.9 ± 0.6 , and 20.4 ± 0.8 d for dairies 1, 2, and 3, respectively. Median days to first insemination were 12, 12, and 16 d for control cows at dairies 1, 2 and 3, respectively. Median days to first insemination were 11, 11, and 17 d for Gpresynch cows at dairies 1, 2, and 3, respectively.

The interaction between treatment and dairy did not ($P = 0.31$) affect hazard of pregnancy; however, hazard of pregnancy was affected ($P = 0.02$) by treatment because Gpresynch cows became pregnant at a faster rate than control cows (AHR = 1.25; CI = 1.04 to 1.50). Mean days from study d 0 to pregnancy were 18.3 ± 0.7 and 15.9 ± 0.7 d for control and Gpresynch cows, respectively ($P = 0.02$; Figure 2). Furthermore, hazard of pregnancy was affected by dairy ($P = 0.05$). Cows at dairy 1 became pregnant faster ($P = 0.01$) than cows at dairy 3 (AHR = 1.33; CI = 1.06 to 1.67). In addition, cows at dairy 1 tended ($P = 0.10$) to become pregnant at a faster rate than cows at dairy 2 (AHR = 1.22; CI = 0.96 to 1.53). Mean days from study d 0 to pregnancy were 15.9 ± 0.7 , 16.7 ± 0.8 , and 19.6 ± 1.0 d for dairies 1, 2, and 3, respectively.

DISCUSSION

In the United States, 93% of dairy herds use estrus detection in their reproductive program as one of the methods to identify cows to be inseminated (NAHMS, 2007). Success of reproductive programs that predominantly use estrus detection for AI depends on facility design and accuracy of detecting cows in estrus. In addition, presynchronization and synchronization programs tailored to maximize insemination based on estrus detection may affect AI submission rate, which influences reproductive efficiency of dairy herds. Presynchronization protocols based on PGF_{2α} before initiation of TAI programs induce luteolysis and ovulation without suppressing estrus expression (Chebel et al., 2013). Nevertheless, the advantage of using PGF_{2α}-based presynchronization methods in programs tailored to inseminate cows in estrus is limited to cyclic cows. Considering that anovular cows may benefit from treatment with GnRH, one of the main purposes of the present experiments was to incorporate a GnRH injection in a PGF_{2α}-based presynchronization strategy to maximize the response of the PGF_{2α} injections in a program focused to inseminate cows based on estrus detection. Furthermore, we hypothesized that treatment with GnRH before initiation of the presynchronization protocol with PGF_{2α} injections may increase P/AI as a result of increased concentration of progesterone before the first PGF_{2α} injection.

Even though overall P/AI did not differ between treatments, multiparous cows (experiment 2) treated with GnRH before the presynchronization protocol became pregnant at a faster rate compared with cows not treated with GnRH. In addition, Gpresynch cows in experiment 1 had greater P/AI than control cows in one of the herds (dairy 1—primiparous cows). On the other hand, the opposite was observed at dairy 2. It is important to note that control cows from dairy 1 had decreased P/AI compared with control cows from dairies 2 and 3, indicating differences in fertility of primiparous cows among herds. The potential causes for differences in fertility of primiparous cows among herds also might have resulted in distinct responses to the GnRH and PGF_{2α} injections. Responses to the hormonal treatments might be influenced by several factors, including management practices, facilities, and percentage of anovular cows. Based on the findings from experiment 1, one could suggest that the treatment with GnRH in primiparous cows was only beneficial in the herd with decreased fertility, potentially with increased prevalence of anovular cows. Unfortunately, cows from dairy 1 were not enrolled in the subgroup of cows in which ovaries were examined by ultrasonogra-

phy, which limits our conclusions. Nonetheless, other authors have demonstrated that incorporating GnRH in presynchronization protocols for first TAI only benefited a subpopulation of cows (Souza et al., 2008). Only primiparous cows benefited from a GnRH-PGF_{2α}-based presynchronization protocol, not multiparous cows (Souza et al., 2008), which are expected to be at a lesser risk for anovular condition. Indeed, Ribeiro et al. (2012) suggested that the lack of benefit of a GnRH-PGF_{2α}-based protocol observed in grazing dairy herds was related to the low proportion of anovular cows (14.1%). Unfortunately, the 2 previous reports did not account for season in their statistical analysis (Souza et al., 2008; Ribeiro et al., 2012), limiting the understanding of how cows responded to the protocols during the summer.

The negative effect of GnRH treatment on overall P/AI of Gpresynch cows from dairy 2 is intriguing (experiment 1—primiparous). Nevertheless, a previous study has shown evidence of a detrimental effect on fertility of cows treated with GnRH before a PGF_{2α}-based presynchronization protocol (Bittar et al., 2014). Treating cows with GnRH at 17 ± 3 and 20 ± 3 DIM resulted in decreased fertility of cows bearing a CL at treatment that failed to ovulate to the GnRH injections (Bittar et al., 2014). Despite the fact that Bittar et al. (2014) administered GnRH earlier postpartum compared with the current study, the differences in P/AI between treatments for primiparous cows from dairy 2 could be explained by the potential poor fertility of cows that failed to ovulate to the GnRH injection.

The increased proportion of cyclic cows at the first PGF_{2α} injection in the Gpresynch treatment indicates that the GnRH treatment was effective in treating anovular condition in a subset of cows. Gumen et al. (2003) demonstrated that the first GnRH injection of the Ovsynch protocol was effective in inducing ovulation of anovular cows; however, P/AI of anovular cows was decreased compared with cyclic cows. In the current study, it is likely that anovular cows that ovulated to the GnRH injection at study d 0, and had subsequent PGF_{2α}-induced luteolysis and detected in estrus, had decreased P/AI compared with cows that were already cyclic. It is possible that we did not detect P/AI differences between treatments during period 2 because a greater proportion of Gpresynch cows that were in anovular condition were induced to ovulate before the first PGF_{2α} injection and were inseminated during period 2. Thus, these cows may have decreased overall P/AI of Gpresynch cows during period 2.

Despite the greater percentage of cows with a CL present at the first PGF_{2α} injection observed in both experiments, the increase in ovulation risk only resulted

in an increased concentration of progesterone in experiment 1 (primiparous cows). A possible explanation for these results could be differences in concentration of progesterone at the time of GnRH treatment, which was lesser in experiment 1 compared with experiment 2. The decreased progesterone concentration might have promoted a greater response to the GnRH injection due to decreased negative feedback of progesterone on the hypothalamus and greater release of LH (Giordano et al. 2012; Pulley et al., 2015). A more plausible explanation for these findings may be related to the design of the experiments. Because treatment with GnRH in experiment 1 was administered after cows were eligible to be inseminated, cows that did not respond to the GnRH injection might have been inseminated between GnRH and PGF_{2α} treatments and a sample at study d 7 was not collected from these cows. Thus, it is likely that an increased proportion of Gpresynch cows from experiment 1 sampled at study d 7 responded to the GnRH injection compared with Gpresynch cows from experiment 2. The collaborating dairies in the current study desired to initiate the presynchronization protocol earlier for multiparous cows than primiparous, but maintain a similar voluntary waiting period (VWP). Because of differences in initiation of protocols relative to the end of the VWP, the authors decided to allocate parity by experiment and conduct the statistical analysis separately to avoid the confounding effects of DIM at initiation of treatment.

Suppression of estrus after administration of GnRH has been well documented (Mendonça et al., 2012; Chebel et al., 2013). In experiment 1 (primiparous cows), GnRH was administered 4 to 10 d after the end of the VWP. Therefore, Gpresynch cows eligible to be inseminated had their expression of estrus suppressed, resulting in treatment differences in the percentage of cows inseminated from study d 0 to 7. In experiment 2 (multiparous cows), GnRH was administered before cows were considered eligible to be inseminated by the farm personnel. Therefore, the pattern in which multiparous cows (experiment 2) were inseminated was not affected by the suppression of estrus from the GnRH treatment to the same extent as the insemination pattern was affected for primiparous cows (experiment 1). The increased percentage of Gpresynch cows having a CL at study d 7 explains the greater proportion of Gpresynch primiparous cows being inseminated after the first PGF_{2α} injection (period 2) compared with control cows. In contrast, in multiparous cows, the difference in the proportion of Gpresynch cows inseminated in period 2 compared with control cows was only observed in dairy 1. These findings support the premise that responses to GnRH and PGF_{2α} treatments were

not similar in dairy 1 compared with dairies 2 and 3. As previously mentioned, cow-related factors might have influenced hormonal responses. Factors not assessed in this study (e.g., BCS and body core temperature) were likely associated with the observed treatment differences across herds. Friedman et al. (2011) had previously reported that the benefit of treating heat-stressed cows with GnRH and PGF_{2α} was limited to subpopulations of cows. Among cows with BCS >2 at 50 to 60 DIM, treatments with GnRH and PGF_{2α} increased P/AI compared with no treatment. Furthermore, the treatments benefited primiparous, but not multiparous cows (Friedman et al., 2011). The previous authors speculated that the lesser milk production of primiparous cows may have resulted in lower body temperature compared with multiparous cows, leading to a better response to the hormonal treatments.

Heat stress has been shown to decrease the expression and duration of estrus in dairy cattle (Younas et al., 1993), justifying why we conducted this study during summer. We attempted to increase expression of estrus in the critical time of the year. The current experiments demonstrate that regardless of the possible decrease in estrus expression during summer caused by heat stress, lactating dairy cows may still be detected in estrus efficiently. The findings from the current study demonstrate that 86.3 and 89.3% of cows from experiment 1 and 2, respectively, were detected in estrus and inseminated before initiation of the TAI protocol. Cartmill et al. (2001) reported that fewer cows were detected in estrus when THI was ≥ 72 . In the current study, the mean daily percentage of hours with THI ≥ 72 was lesser for dairy 1 than dairies 2 and 3, 50.4 and 70.9%, respectively. Although cows from the 3 herds were exposed to heat stress conditions during the experiments, cows from dairy 1 might have experienced a lesser extent of heat stress compared with cows housed in dairies 2 and 3, despite the differences in heat abatement strategies used in the dairies. It is possible that the increased severity of heat stress for cows located in dairies 2 and 3 might have further affected the response to GnRH treatment via altered GnRH-induced LH release. Cows under chronic heat stress conditions had lesser peaks of GnRH-induced LH (Gilad et al., 1993). In addition, responses to PGF_{2α} treatments may have been decreased in cows located in dairies 2 and 3 because of the exacerbated heat stress. Luteolysis after PGF_{2α} is less likely to occur during summer than during moderate or cold weather (Stevenson et al., 2012). Thus, luteolysis is presumably affected by the severity of heat stress.

In conclusion, results from the current experiments collectively suggest that using a GnRH-PGF_{2α}-based

presynchronization protocol has benefits in some herds compared with a PGF_{2α}-based presynchronization protocol during summer heat stress. The current study demonstrated that treatment with GnRH induced ovulation of cows in anovular condition. In addition, treatment with GnRH 7 d before initiating a PGF_{2α}-based presynchronization protocol results in a greater proportion of cows bearing a CL at the first PGF_{2α} injection, which may result in a greater percentage of cows to be inseminated earlier in lactation, and consequently, increased AI submission rate. Nevertheless, if GnRH is administered after cows become eligible to be inseminated, estrus is suppressed and it may negatively influence insemination rate. Herds with efficient estrus detection could benefit from a GnRH-PGF_{2α}-based presynchronization protocol initiated before the end of the VWP, which can potentially decrease days open by inseminating a greater proportion of cows earlier in lactation. Differences in treatments across herds are an indication that cow-related factors potentially affect the responses of including a GnRH injection before starting the presynchronization protocol. The percentage of anovular cows likely influences the response to GnRH and PGF_{2α} injections and should be considered before implementation in dairy herds. Further, the severity with which heat stress affects cows might affect responses to the hormonal treatments, thus affecting P/AI and insemination risk.

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